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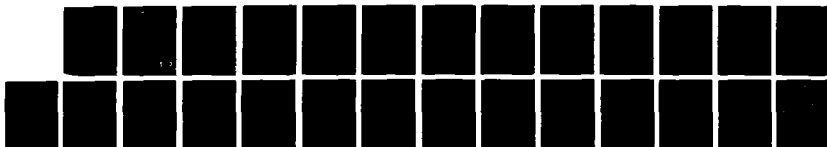
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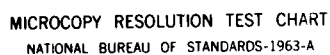
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NEW APPROACHES TO ATTENUATED HEPATITIS A VACCINE DEVELOPMENT:
CLONING AND SEQUENCING OF CELL-CULTURE ADAPTED VIRAL cDNA

ANNUAL REPORT

Stanley M. Lemon, M.D.

October 13, 1986

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A secondary goal of the work in progress is to document the existence of strain-specific differences among various isolates of HAV, and to develop methods for the identification of specific HAV strains. Substantial genomic heterogeneity has been defined among several strains of HAV using cDNA-RNA hybridization under conditions of varied stringency; sequence variation is greatest in the P1 and P2 genomic regions, and substantially less in the non-translated 5' leader sequence. These studies appear to define the PA21 and PA33 strains of HAV as unique from human strains; these strains probably represent indigenous virus of the owl monkey.



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SUMMARY

The goal of this research contract is to develop an improved understanding of the biological basis of cell culture adaptation and attenuation of HAV, as this may ultimately lead to a rational approach to development of an effective attenuated vaccine. Accordingly, a cDNA genomic library spanning over 98% of the genome of a plaque-purified, cell culture-adapted variant of HM175 strain HAV (p17 ca-HM175) has been prepared in vector pBR322. Selected cDNA clones have been subcloned into the M13mp19 vector, and subjected to sequencing by the dideoxynucleotide method. In approximately 1 kb of the genome sequenced thus far, a total of 3 nucleotide substitutions have been identified in comparison with the known sequence of wild-type virus (wt-HM175); one of these mutations is silent, while the other two result in amino acid alterations in virus proteins. Completion of these studies will lead to an improved understanding of the biological basis of adaptation of virus to cell culture, and possibly attenuation.

A secondary goal of the work in progress is to document the existence of strain-specific differences among various isolates of HAV, and to develop methods for the identification of specific HAV strains. Substantial genomic heterogeneity has been defined among several strains of HAV using cDNA-RNA hybridization under conditions of varied stringency; sequence variation is greatest in the P1 and P2 genomic regions, and substantially less in the non-translated 5' leader sequence. These studies appear to define the PA21 and PA33 strains of HAV as unique from human strains; these strains probably represent indigenous virus of the owl monkey.

FOREWORD

The investigator has abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (April 1982) and the Administrative Practices Supplements.

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INTRODUCTION

Type A viral hepatitis remains a serious threat to the health and combat effectiveness of the American soldier during times of conflict or deployment to regions outside of North America and Western Europe (1). The capacity of this virus for epidemic spread, the tendency to cause symptomatic infections in non-immune adults, and the relatively long duration of illness and extended convalescence necessary in most cases of acute hepatitis A all contribute to the threat posed by this virus (2). During the second World War, acute hepatitis, probably due largely to hepatitis A virus (HAV), was a significant problem for American soldiers deployed in the Middle East and Western Pacific theaters (1). Acute hepatitis remained an important problem during the Korean conflict. More recently, outbreaks of hepatitis A among U.S. military units have at times forced the cancellation of major training activities and have interfered with normal military operations (3). These outbreaks, occurring during peace time under presumably optimal sanitation conditions, serve to underscore the significant threat posed by this virus in times of conflict. Compared with the military forces of many other nations, U.S. military personnel are at particular risk for hepatitis A infection because they generally have a very low prevalence of antibody (1). For these reasons, there is an urgent need to develop a reasonably priced, safe and effective vaccine for hepatitis A.

Several approaches to development of a vaccine for hepatitis A virus have been proposed. These have included the development of inactivated, formalin-treated virus vaccines produced in cell culture, the development of a live, attenuated virus vaccine, and newer types of vaccines based upon recombinant antigens or synthetic peptides. The relative merits of these different approaches and current developments in this general field have been recently reviewed by the principal investigator (2). A prototype inactivated vaccine has been produced recently from virus grown in BS-C-1 cells at the WRAIR. This vaccine consisted of a formalin-inactivated crude cell extract. This model vaccine, which could not be evaluated in man given the cell type used for virus propagation, was shown to be immunogenic and protective in owl monkeys against virulent virus challenge (4). More recently, a similar vaccine has been produced in MRC-5 cells and approved for phase 1 trials in man. While an exciting development in this area, the immunogenicity of this vaccine has unfortunately proven to be disappointing in man. A highly purified, inactivated cell culture-derived vaccine has been produced by the Merck Institute for Therapeutic Research and has been successfully evaluated in primates. This vaccine has not yet been tested in humans. While perhaps offering the most rapid path to an operational vaccine, the eventual costs of inactivated vaccines produced in cell culture are uncertain but are likely to

be high given the relatively low yields of this virus in cell culture.

While attention has also focused on development of a vaccine using recombinant DNA technology, there has been no reported success in expression of hepatitis A antigen in any recombinant system. Such efforts will be hampered, as they have been with poliovirus, by the fact that the critical neutralization immunogens of HAV appear to be assembled and conformationally dependent. Similarly, while neutralizing antibody has been elicited by immunization with certain synthetic peptides, such immunogens have been relatively weak and induced antibody titers low. Recent studies within the principal investigator's laboratory have suggested that HAV has a single, highly immunodominant neutralization immunogenic site (5) and thus might be quite different from the well characterized poliovirus type 1 and human rhinovirus 14 viruses, which each have several distinct immunogenic sites (6-8). HAV variants (HM175 strain of HAV) that are resistant to neutralizing monoclonal antibodies ("neutralization escape mutants") have been plaque purified (5). Resistance to neutralization was found to be associated with decreased affinity of antibody for cognate epitopes on the viral surface, and we are currently attempting to characterize nucleotide substitutions (and thus by inference amino acid changes) accompanying neutralization resistance. This approach will lead to the identification of the amino acid sequences comprising the neutralization immunogenic site(s) of HAV, and may therefore suggest improved approaches to construction of immunogenic peptide vaccines.

Despite these recent advances, development of a live, attenuated vaccine for hepatitis A remains an important alternative approach. For several reasons, including cost, ease of administration, and possibly better protection, an attenuated vaccine is particularly attractive (2). Studies in several laboratories have demonstrated that adaptation and passage of HAV in cell culture, which is a step-wise process resulting in improved yields and more rapid replication of virus, is also associated with loss of virulence for primates (9,10). Taking this approach, investigators at the Merck Institute for Therapeutic Research have developed a panel of cell-culture adapted HAV variants (CR326 strain) which have been shown to have decreased but varying degrees of virulence in two species of primates (chimpanzees and marmosets) (10). Several selected variants have also been tested in small numbers of humans. The most attenuated of these variants (according to results in primate challenge studies) were found to be non-infectious in man. Less attenuated variants have been shown to be relatively avirulent in man, although occasionally associated with low grade liver enzyme elevations. Administration of an intermediate variant (F' variant of CR326) was not associated with liver

enzyme elevations, but was not immunogenic in all recipients (P. Provost, personal communication). Thus, although results have been promising to date, an acceptable balance of attenuation (loss of ability to induce hepatic injury) and immunogenicity (infectivity) has not yet been reached (2). Similar results have been obtained in non-human primates with cell-culture adapted variants of HM175 strain HAV isolated at the National Institute of Allergy and Infectious Diseases (NIAID). Other problems with development of an attenuated vaccine center on the probability of shedding of attenuated virus in feces, and the possibility of reversion of virus to virulence. Although shedding has been demonstrated with such cell culture-adapted variants, it has not been possible to determine the rate of reversion to virulence, due to the lack of an in vitro marker of attenuation. These issues have been discussed in a recent review of this field by the principal investigator (2).

The major specific aim of work supported under this contract has been to determine the biologic basis of adaptation of HAV to cell culture. An understanding of the genetic changes occurring in HAV during adaptation to cell culture may also shed light on the biological basis of attenuation of this virus as cell culture adaptation and loss of virulence appear to be coincident events. Inasmuch as adaptation to cell culture and loss of virulence are stepwise processes, multiple mutations will undoubtedly be found in any direct comparison of wild-type (wt) and culture-adapted (ca) variants. A second major goal of work supported under this contract has been the development of methodologies capable of distinguishing individual strains of HAV. Rapid and specific strain identification will be necessary for the practical application and study of HAV vaccines when effective preparations become available.

EXPERIMENTAL APPROACH

Work completed to date has involved primarily the HM175 strain of HAV. This strain of virus, originally recovered from a naturally infected human in Australia (11), was molecularly cloned by workers at the NIAID after three sequential experimental passages in marmosets (12). The complete nucleotide sequence of this wt-HM175 has been deduced and has been provided to the principal investigator by Dr. John Ticehurst of the NIAID. The immediate objective of work supported under this contract has been the molecular cloning of RNA from a variant of HM175 virus which is derived from the marmoset-passaged virus described above and which is highly adapted to cell culture (ca-HM175). A comparison of the sequence of virion RNA from wt-HM175 and ca-HM175 variants will provide information on specific virus proteins undergoing mutation during adaptation to cell culture, and will also reveal whether cell culture adaptation involves mutations in non-translated regions of the genome. The

biological significance of identified mutations will be ascertained by comparison of the ca-HM175 sequence with that of other ca-HM175 variants that were independently isolated and adapted to cell culture. Such variants are currently being studied in laboratories at the NIAID and the Fairfield Hospital, Melbourne, Australia. An ultimate goal of this general approach is the construction of full-length, infectious cDNA constructs or RNA transcripts of cDNA that could then open the way to site-directed mutagenesis of the virus genome.

With regards to identification of individual strains of hepatitis A virus, initial studies have focused on the application of cDNA-RNA hybridization to the detection of genomic differences among different strains of HAV. These studies have demonstrated that the greatest degree of sequence variation appears to lie in the P1 (capsid-encoding region) of the genome, and that the non-translated 5' portion of the genome is most highly conserved (13). Direct RNA sequencing, using primer extension of virion RNA with specific oligodeoxynucleotide primers and the dideoxynucleotide sequencing technique is currently being explored as a means of specific strain identification.

RESEARCH PROGRESS

1. Molecular Cloning and Sequence Analysis of ca-HM175 virion RNA

Virus HM175 virus was isolated from marmoset liver in primary African green monkey kidney cells, and after ten passages in these cells was adapted to growth in continuous B-SC-1 cells as described (14). This virus was twice plaque-purified using a modification of the radioimmunofocus method described recently (15). cDNA clones were derived from RNA of virus that had undergone a total of 17 passages in cell culture (10p AGMK, 7p BS-C-1).

Isolation of viral RNA Two approaches were taken for production of viral RNA. First, total cellular messenger RNA was taken from infected single roller bottle cultures and used for synthesis of cDNA. Quantitative molecular hybridization studies demonstrated that less than 0.1 percent of cellular message was HAV specific. For this purpose, an 850 cm² roller bottle culture of confluent BS-C-1 cells was inoculated with plaque-purified caHM175 and held for 17 days at 35°C. Actinomycin D was added to the medium on day 15 at a concentration demonstrated to reduce cellular RNA synthesis by >95%. Total cellular RNA was extracted by the guanidinium isothiocyanate-caesium chloride method, and the polyadenylated fraction was selected by passage over an oligo(dT)-cellulose column. Alternatively, as a second approach virion RNA was prepared from gradient-purified virus harvested from the cellular fraction of 4 similarly infected roller bottle

cultures. Viral RNA was prepared by SDS-proteinase K digestion of purified virus, followed by phenol-chloroform extraction. RNA prepared by either method was precipitated in ethanol and stored at -70° C.

Molecular cloning of virus cDNA in vector pBR322 Following first-strand cDNA synthesis, cDNA-RNA hybrids were cloned directly into the vector pBR322 as this approach had proven very successful with poliovirus type 3 and human rhinovirus type 14 RNAs (16). This approach is likely to include 5' terminal sequences normally lost during "hairpin" priming of second strand cDNA synthesis. Potential disadvantages include lower transformation efficiency (cDNA-positive clones per ug cDNA) and possibly greater potential for rearrangements of nucleic acid during the cloning process. RNA (either total cellular polyadenylated RNA, or RNA extracted from purified virions) was size-selected by ultracentrifugation in neutral sucrose gradients. Gradient fractions containing full-length genomic RNA were identified by Northern blot analysis using cDNA probes prepared as described previously (17). First-strand cDNA was reverse-transcribed from viral RNA using AMV reverse transcriptase essentially as described by Ticehurst *et al* (12). Both oligo(dT)₁₂₋₁₈ and random calf thymus DNA oligonucleotide fragments were used as primers in separate experiments. cDNA-RNA hybrids were extracted with phenol-chloroform, passed through a G-100 Sephadex column, and precipitated in ethanol. Hybrid molecules were directly dC-tailed with terminal deoxynucleotidyl transferase, ethanol-precipitated, and annealed to plasmid pBR322 DNA which had been linearized by restriction at the *Pst*I site and tailed with dG. This recombinant DNA was used to transform competent *E. coli* HB101 cells. Resulting tetracycline-resistant colonies were screened by *in situ* colony hybridization using a mixture of cDNA probes spanning most of the HAV genome. These cDNA probes were derived from plasmids provided by Dr. John Ticehurst of the NIAID, and were prepared by random-priming of isolated insert DNA fragments as described previously (17). In several cloning experiments, over 200 *E. coli* clones containing plasmids with stable HAV inserts were isolated.

Characterization of ca-HM175 cDNA clones The length of the insert fragment in selected clones was determined by agarose gel electrophoresis of *E. coli* plasmid DNA "mini-preps". The genomic location of insert fragments was characterized initially by slot-blot hybridization of plasmid DNA using cDNA probes representative of specific regions of the wt-HM175 genome. These studies suggested the presence of substantial rearrangements (i.e., deletions) of cDNA in only one of these clones (as indicated by hybridization with probes derived from noncongruent regions of the wt-HM175 genome). Clones with overlapping insert fragments were further identified by slot-blot hybridization using insert fragments from newly derived ca-HM175 cDNA clones as

probes, and by selected mapping of restriction endonuclease sites. A total of 9-10 clones were determined to have overlapping insert fragments spanning most of the ca-HM175 genome (Figure 1). As expected, clones derived from cDNA synthesized with oligo(dT) were for the most part restricted to the 3' region of genome. Although not yet determined, it is likely that most if not all of these clones contain the 3' terminus (poly-A tract). Clones derived from first-strand cDNA synthesis using random primers were more generally distributed over the length of the genome. Preliminary sequence analysis (see below) suggests that sequences within 50 bases of the 5' end of the genome are included in the plasmid p_{HAV} ch⁸⁸.

Sequence analysis of ca-HM175 cDNA Insert fragments from several of the clones shown in Figure 1 have been subcloned into the vector M13mp19 and subjected to shotgun sequencing using the dideoxynucleotide method of Sangar et al. To date, approximately 1 kb of the genome has been reliably sequenced (Figure 2). A comparison of these sequences with wt-HM175 cDNA sequences has thus far demonstrated the existence of three mutations (Figure 3), one of which is silent and does not encode an alteration in primary protein structure. This work remains in progress and will form a major portion of work to be completed during the second year of this contract.

2. Identification and Characterization of HAV Strain Differences.

cDNA-RNA Hybridization Major differences in the genomic sequences of three distinct strains of HAV have been detected by cDNA-RNA hybridization using conditions of varied stringency (13). Five different cDNA fragments (12) spanning various regions of the genome were used as hybridization probes. These included probes 1307-2 (extending from nucleotide position 162 to 849), 1307-1 (nucleotide 1001-2900), 228 (nucleotide 2450-4600), 207-2 (nucleotide 5550-6062) and 207-1 (nucleotide 6063-7478) (Figure 1). ³²P-labelled probes were synthesized from denatured insert DNA fragments by random priming with calf thymus oligodeoxynucleotides and *E. coli* DNA polymerase I (15).

The strains used in these studies are shown in Table 1. All three strains were studied after adaptation of virus to cell culture. For the purpose of comparing hybridization results between different HAV strains, the amount of each virus blotted was adjusted so as to achieve an equivalent signal intensity with the hybridization probe derived from the 5' end of the genome (probe 1307-2) under standard stringency conditions. These conditions included hybridization at 42 C in the presence of 50% formamide, 1X Denhardt solution, 5X SSC, 0.1% SDS and denatured calf thymus DNA at 100 ug/ml. Probes were boiled for 3 min immediately before dilution into hybridization solutions (2x10⁶ cpm/ml). Hybridization was carried out for 22-36 hrs, following

which the nitrocellulose was washed twice with 2X SSC, 0.1% SDS at room temperature for 15-30 min per wash and twice with 0.1X SSC, 0.1% SDS at 52 C for 15-30 min per wash (final wash). Conditions of reduced stringency included hybridization in 40% formamide at 42 C followed by a final wash at 42 C. In some experiments, hybridization stringency was increased by raising the final wash temperatures to 62 C or 72 C. Autoradiography was carried out using Kodak XAR-5 film and X-Omatic intensifying screens (Eastman Kodak Co., Rochester, NY). Films were developed after exposure for 1-10 days at -70°C.

These studies demonstrated, surprisingly, that cDNA clones derived from most of the P1, capsid-encoding region of the HM175 genome did not hybridize under "standard" conditions with RNA derived from the PA21 virus. In contrast, cDNA derived from the non-translated, 5' leader segment of HM175 RNA appeared to hybridize as efficiently to PA21 virus RNA as to HM175 RNA. cDNA probes derived from the 3' region of the HM175 genome were intermediate in their ability to hybridize to PA21 virus RNA. These results are depicted in Figure 4. In contrast to the results obtained with PA21 virus, there was no apparent difference in the ability of HM175 cDNA probes to hybridize to GR8 and HM175 viruses.

Because the results shown in Figure 4 suggested that HM175 and GR8 viruses shared significantly greater homology than HM175 and PA21 viruses, it was of interest to examine other human strains of virus. Wild-type viruses were used for this analysis; HM175 virus was purified from feces of an experimentally infected owl monkey (virus passed once in the owl monkey), while GR8, LV374 and MS1 viruses were purified directly from human specimens. The blotted dilutions of virus were standardized as before to yield equivalent hybridization signals with probe 1307-2, and replicate blots were hybridized against the five HM175-derived cDNA probes under standard stringency conditions. These experiments revealed no detectable differences between four wild-type human viruses (HM175, GR8, LV374 and MS1) (data not shown) or between wild-type and cell culture-adapted virus variants (HM175 and GR8) under standard hybridization conditions. The absence of detectable differences among these human strains contrasts with the results obtained with the owl monkey-derived PA21 virus, and suggests that PA21 and the epizootiologically-related PA33 viruses are unique viruses indigenous to that primate species. Hybridization of selected HM175 cDNA fragments to RNA from other human strains of HAV followed by high stringency washes as described above was able to distinguish between different virus strains, as described in greater detail in the manuscript appended to this Annual Report (13).

Solid-phase monoclonal radioimmunoassay Because genomic variation between HM175 and PA21 viruses was maximal within the

P1 region of the genome (see above), the antigenic characteristics of these viruses were examined by determining the ability of several murine monoclonal antibodies to bind to specific neutralization epitopes on each virus strain. Monoclonal antibodies included K3-4C8, K3-2F2 and K2-4F2, which were kindly provided by I.D. Gust and A.G. Coulepis of the Fairfield Hospital, Melbourne, Australia, and B5-B3 which was the generous gift of R.S. Tedder of the Middlesex Hospital, London, U.K. These monoclonal antibodies have high titer neutralizing activity and are directed against unique, but closely positioned or overlapping epitopes on the HM175 virus capsid (5).

For detection of unique HAV neutralization epitopes on individual strains of virus, radiolabelled murine monoclonal antibodies to HAV were allowed to bind to virus previously captured onto the solid-phase support with polyclonal antisera. Briefly, 100 μ l aliquots of a 1:1000 dilution of human convalescent serum (JC), diluted in 50 mM carbonate buffer, pH 9.6, were incubated in wells of a flexible polyvinyl chloride microtiter plate for 4 hrs at 37 C. The wells were washed three times with PBS containing 0.05% Tween-80 (PBS-T), and 50 μ l aliquots of gradient-purified HAV, diluted in PBS, were placed in each well. After incubation overnight at 4 C, the wells were washed three times with PBS-T, and 50 μ l of a solution containing radiolabelled monoclonal antibody (approximately 200,000 cpm) was added to each well. The microtiter plates were placed at 4 C for 4 hrs, washed 5 times with PBS-T, and individual wells were cut out and placed in an automatic gamma counter (LKB Rackgamma, LKB Instruments, Turku, Finland) to determine residual bound radioactivity. Monoclonal antibodies for these experiments were purified from murine ascitic fluids by precipitation in 50% ammonium sulfate, and were labelled by a modification of the chloramine T method after extensive dialysis against PBS. The degree to which radiolabelled monoclonal antibodies bound to different quantities of virus fixed to a solid-phase support was assessed as shown in Figure 5.

Neutralization epitopes recognized by all four monoclonal antibodies were present on both viruses. Furthermore, there was little difference in the relative avidity of the radiolabelled antibodies for the two virus preparations, with the exception of B5-B3 which bound more avidly to HM175 virus. Thus these experiments confirmed that only a minor degree of antigenic variation exists between these two strains of virus, despite the poor homology evident within the P1 region which encodes the capsid proteins. At present, a similar analysis is being carried out with a variety of other HAV strains and should provide a detailed picture of antigenic variation among diverse isolates of HAV.

Because of the poor genome homology evident between the P1 regions of RNA from these viruses, despite the fact that in both viruses this region encodes highly conserved antigenic structure(s), we have begun efforts directed at the molecular cloning of cDNA derived from PA21 virus. A comparison of PA21 and HM175 RNA sequences will provide important information concerning the relatedness of these HAV strains and may reveal highly conserved regions encoding the conserved epitopes. This analysis will provide an alternate approach to identification of critical neutralization immunogenic sites on the virus surface.

Direct sequencing of viral RNA by primer extension

Substantial effort has been directed at direct sequencing of viral RNA by primer extension of RNA using specific synthetic oligodeoxynucleotides complementary to the VP1 (i.e. P1) region of the HM175 virus genome (see Table 2). These synthetic primers were prepared by the Oligonucleotide Synthesis Facility of the Department of Microbiology and Immunology, UNC-CH, under the direction of Dr. Clyde Hutchison. Two approaches have been taken to primer extension of viral RNA derived from infected roller bottle cultures of BS-C-1 cells. In each case, the amount of template RNA used in individual reactions has been on the order of 10 ng as determined by quantitative hybridization using plasmid DNA standards. This is substantially less than the quantities of RNA that generally have been used in similar studies with other picornaviruses. We feel that it is important to be able to carry out this analysis with minimal amounts of viral RNA if it is to be applied to fecally-derived, non-cell culture-adapted virus, or to low passage virus isolates.

The first approach has been to 5' end-label synthetic oligonucleotide primers to high specific activity using [³²P]-ATP and T4-polynucleotide kinase, and to extend labelled primers against viral RNA using AMV reverse transcriptase in the presence of appropriate concentrations of dideoxynucleotide triphosphates (6,7). With this approach, we have been able to generate readable sequencing "ladders" following separation of the reaction products on 6% polyacrylamide gels. These studies have permitted the sequencing of up to 50 bases from a single primer. We are now engaged in studies with a second method involving the use of non-labelled primers and a primer-extension reaction mix containing deoxynucleotide triphosphates labelled to very high specific activity with [³⁵S]. Overall, this approach appears encouraging but is still under development. If successfully developed, it should permit relatively rapid strain identification using very small amounts of starting virus.

CONCLUSIONS

The following progress has been accomplished during the first year of this contract:

1. A cDNA genomic library spanning over 98% of the genome of a plaque-purified, cell culture-adapted variant of HM175 strain HAV (p17 ca-HM175) has been prepared in vector pBR322.
2. Selected cDNA clones have been subcloned into the M13mp19 vector, and subjected to sequencing by the dideoxynucleotide method.
3. In approximately 1 kb of the genome sequenced thus far, a total of 3 nucleotide substitutions have been identified in comparison with the known sequence of wild-type virus (wt-HM175); one of these mutations is silent, while the other two result in amino acid alterations in virus proteins. Completion of these studies will lead to an improved understanding of the biological basis of adaptation of virus to cell culture, and possibly attenuation.
4. Substantial genomic heterogeneity has been defined among several strains of HAV using cDNA-RNA hybridization under conditions of varied stringency; sequence variation is greatest in the P1 and P2 genomic regions, and substantially less in the non-translated 5' leader sequence. These studies appear to define the PA21 and PA33 strains of HAV as unique from human strains; these strains probably represent indigenous virus of the owl monkey.
5. Monoclonal radioimmunoassays specific for neutralization epitopes of HAV have been developed and are being applied to characterization of antigenic variation among HAV strains. Despite substantial genetic divergence between HM175 and PA21 viruses, these strains of HAV retain well conserved neutralization epitopes.
6. A number of synthetic oligodeoxynucleotides complementary to the VP1-encoding region of HM175 virus have been synthesized, and are being used in primer-extension sequencing of viral RNA as a direct approach to identification of specific HAV strains.

Table 1. Infectivity and antigen content of standardized dilutions of cesium chloride gradient-purified, cell culture-derived HAV strains studied for genomic heterogeneity by molecular hybridization with cDNA probes.

| Virus | Infectivity titer | Radioimmunoassay titer |
|-------|-------------------|------------------------|
| HM175 | 5.79 ^a | 1:3.5 ^b |
| GR8 | 5.92 | 1:2.6 |
| PA21 | 5.95 | 1:2.0 |

- (a) Log₁₀ radioimmunofocus-forming units/ml (15).
 (b) Radioimmunoassay titer of original gradient fraction multiplied by the dilution factor.

Table 2. Synthetic oligodeoxynucleotide primers complementary to VP1-encoding regions of the HM175 virus genome.

| MAP POSITION | | | | | n | %GC |
|--------------|-------------------|--|----------|--|----|-----|
| 2366 | sequence oligo | 5' A ACA ATT GAG GAT C 3' T TGT TAA CTC CTA G | 3' 5' | | 14 | 36% |
| 2436 | sequence oligo | 5' AGA CAT ACA TCA GAT 3' TCT GTA TGT AGT CTA | 3' 5' | | 15 | 33% |
| 2556 | sequence oligo | 5' TCT AAT CCT CCT CAT GGT TT 3' AGA TTA GGA GGA GTA CCA AA | 3' 5' | | 20 | 43% |
| 2797 | sequence oligo | 5' AC ATT CAG ATT AGA T 3' TG TAA GTC TAA TCT A | 3' 5' | | 15 | 27% |
| 2949 | sequence oligo | 5' TAT TTG TCT GTC ACA 3' ATA AAC AGA CAG TGT | 3' 5' | | 15 | 30% |
| 3174 | sequence oligo | 5' TAT GCT CAG GAA GA 3' ATA CGA GTC CTT CT | 3' 5' | | 14 | 42% |

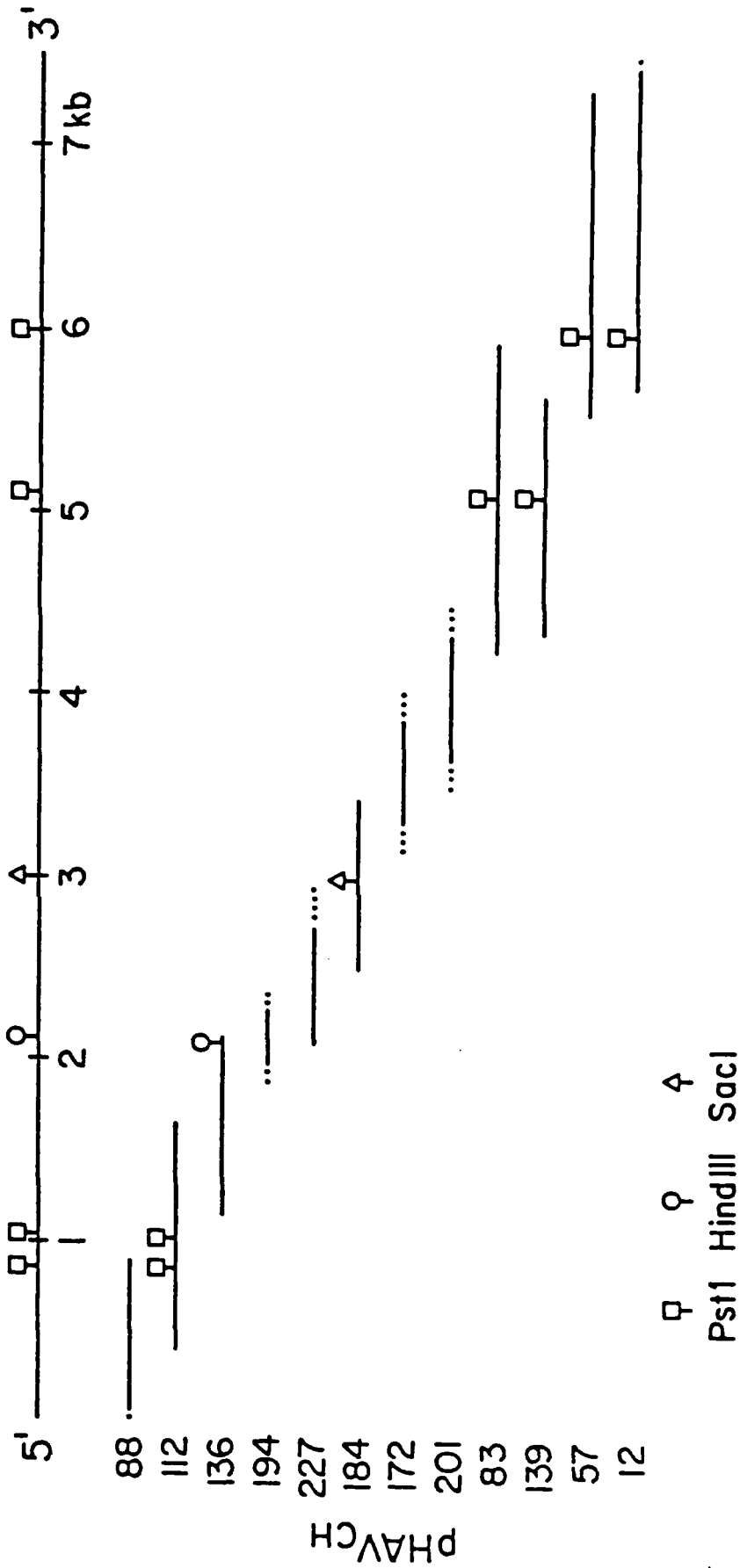


Figure 1. Map of ca-HM175 cDNA clones. Insert regions were mapped by hybridization with cDNA clones derived from wt-HM175 virus. Regions of overlap were established by hybridization and limited restriction mapping.

Figure 2. Nucleotide sequence of insert fragment from clone
 PHAV_{ch} 136 (top sequence) and PHAV_{ch} 12, small fragment (12-2)
 (bottom sequence) following PstI digestion.

TTTCCAACAGGGGGGATTGATCTGTGCTATGGTTCCCTGGT
 GACCAGAGCTATGGTTCTATAGCATCATTGACTGTTTATCCTCATGGTTTGTAAATTGC
 AATATTACAATGTGGTTAGAATAAAGGTTCCATTTATTTACACAAGAGGTGCTTACCAC
 TTTAAAGATCCACAATACCCAGTTTGGGAATTGACAATTAGAGTTTGGTCAGAATTAAAT
 ATTGGGACAGGAACCTTCAGCTTATACTTCACTCAATGTTTTAGCTAGATTTACAGATTG
 GAGTTGCATGGATTAACTCCTCTTTCTACACAATGATGAGAAATGAATTTAGGGTCAGT
 ACTACTGAGAATGTGGTGAATCTGTCAAATTATGAAGATGCAAGAGCAAAGATGTCTTTT
 GCTTTGGATCAGGAAGATTGGAAATCTGATCCGTCCCAGGGTGGTGGGATCAAATTACT
 CA. TTTACTACTTGGACATCTATTCCAACCTTTGGCTGCTCAGTTTCCATTTAATGCTTCA
 GACTCAGTTGGTCAACAAATTAAAGTTATTCCAGTTGACCCATATTTTTTCCAAATGACA
 AATACAAATCCTGACCAAAAATGTATAACTGCTTTGGCTTCTATTTGTGAGATGTTTTGT
 TTTTGGAGAGGAGATCTTGTCTTTGATTTTCAATTTTTTCCACCAAAATATCATTGAGGT
 AGATTACTGTTTTGTTTTGTTCTTGGCAATGAGCTAATAGATGTTTCTGGAATCAC-TTA
 AGCAAGCACTACTGCTCCTTGTGCACTAATGGATATTACAGGAGTGCAGTCAACTTTGAG
 ATTTCTGTGTTCCCTGGATTTCTGACACTCCTTACAGAGTGAACAGGTATACAAGTCAGCA
 CACTTCAGAAAGGTGAGTACACTGCCATTGGGAAGCTTATTGTGTATTGTTATACAGATC
 TCCTCTCCAAA

GGAAAATTAATCATGGTTTTATCAATGTGATGATAAATGGGACTCTTTATAAAAAGCGTT
 TTGGAGACCACATTCATTGAACACTGAGTAACTCCACTTTTATAATTCTCTGACTTTCA
 ATTTTCTTATCAATATTTTGGAACTTTCTTGAGTAACCAATTTTGCACAAGAATTGAA
 TTTCTCCAGCAACACATGGATGCCCAAGATTGCATTCTGTA

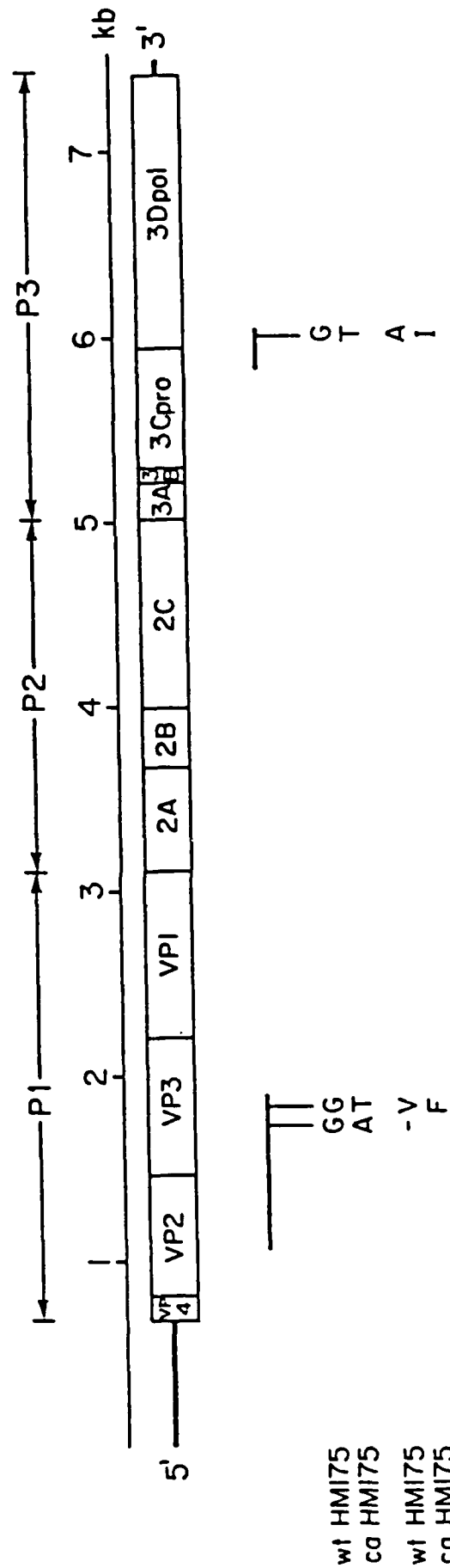


Figure 3. Genomic map of HAV showing (top to bottom) major genomic regions (P1, P2, P3), size scale, putative peptide cleavage sites (provided by J. Ticehurst, NIAID), ca-HM175 regions sequenced thus far, differences in the nucleotide sequences of wt and ca-HM175, and respective amino acid substitutions.

wt HM175
ca HM175
wt HM175
ca HM175

Figure 4. RNA from three cell-culture adapted strains of HAV (HM175, GR8 and PA21) was allowed to hybridize to five cDNA probes (1307-2, 1307-1, 228, 207-2, 207-1) derived from different regions of the HM175 virus genome. Five replicate blots were prepared by directly blotting a series of four four-fold dilutions of purified virus onto the nitrocellulose. The highest dilution of virus blotted was adjusted so as to yield comparable hybridization signals with each virus when hybridized with probe 1307-2 (see Table 1). The genomic map of HM175 virus is depicted at the top of the figure, along with the putative location of the regions encoding the capsid polypeptides (VP1-4) and the regions represented by the five cDNA probes. Hybridization was for 22 hours at 42 C in the presence of 50% formamide; the final wash temperature was 52 C. Film was exposed for 24 hours.

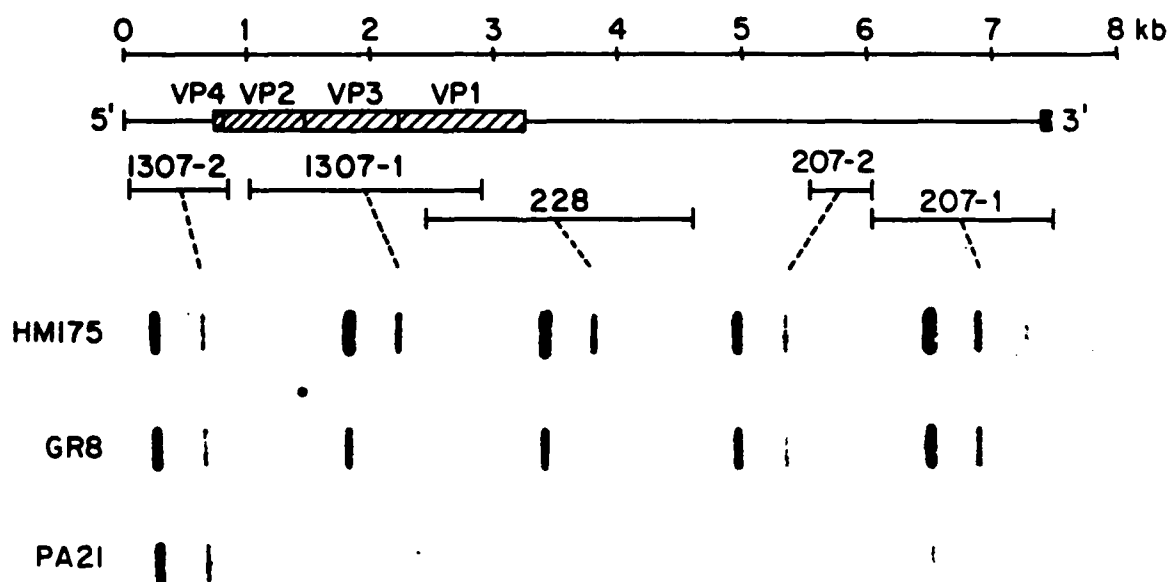
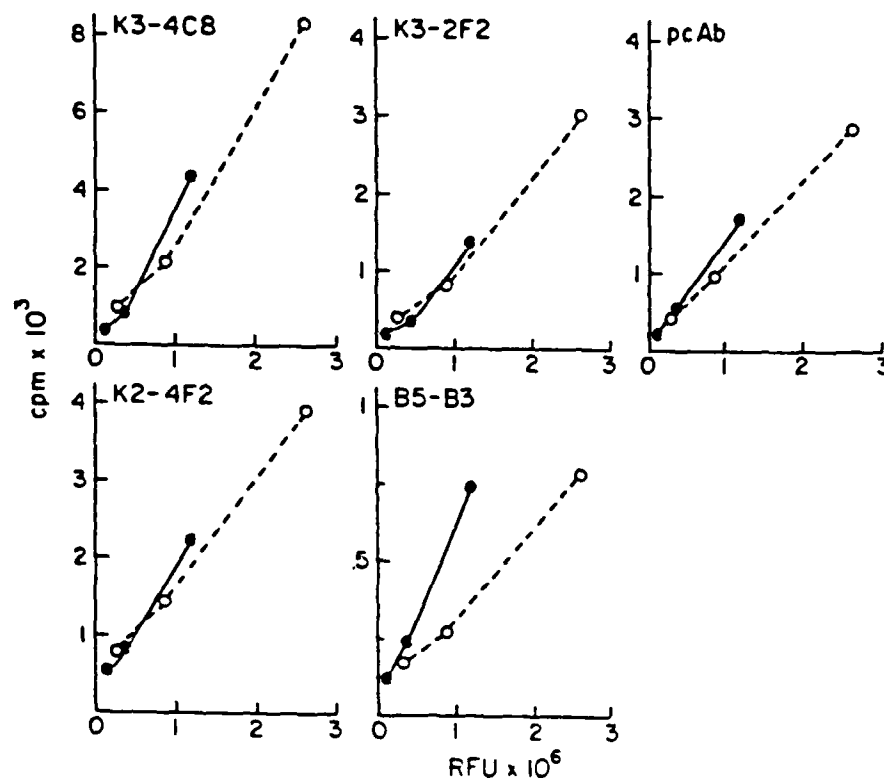


Figure 5. Monoclonal antibody analysis of HM175 and PA21 viruses. Dilutions of gradient-purified virus were allowed to bind to polyclonal human antibody fixed on a solid-phase support. Immobilized virus was then exposed to radio-labelled monoclonal (K2-4F2, K3-2F2, K3-4C8 and B5-B3) or polyclonal (pcAb) antibodies. Virus quantities present in each dilution were estimated by radioimmunoassay of the original gradient fractions. Solid lines represent HM175 virus, dashed lines PA21 virus.



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PUBLICATIONS SUPPORTED BY THIS CONTRACT

1. Lemon, S.M., S-F. Chao, R.W. Jansen, L.N. Binn, and J.W. LeDuc. 1986. Genomic heterogeneity among human and non-human strains of hepatitis A virus. Submitted for publication (J Virology).

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